Reference: *Biol. Bull.* **181**: 189–194. (August, 1991)

Vanadobin, a Vanadium-Binding Substance, Extracted from the Blood Cells of an Ascidian, Can Reduce Vanadate(V) to Vanadyl(IV)

HITOSHI MICHIBATA[1,*], AKEMI MORITA[1], AND KAN KANAMORI[2]

[1]*Biological Institute and* [2]*Department of Chemistry, Faculty of Science, Toyama University, Gofuku 3190, Toyama 930 Japan*

Abstract. Ascidians specifically accumulate high levels of vanadium from seawater in their blood cells. Almost all of the vanadium is present in a reduced form in the blood cells, although the metal exists in a +5 oxidation state in seawater. It has, therefore, been assumed that agents that cause the reduction of vanadate(V) to vanadyl(IV) must be present within ascidian blood cells. In this regard, we have extracted a vanadium-binding substance, which we have called vanadobin, from the vanadocytes of ascidians. We examined whether vanadobin is involved in the reduction of vanadate(V) accumulated from seawater. Data obtained by spectrophotometry and ESR spectrometry revealed that not only a crude homogenate of vanadium-rich blood cells but also a purer form of vanadobin eluted from a column of Sephadex G-15 could reduce vanadate(V). Our experiments demonstrate that vanadobin, a vanadium-binding substance extracted from ascidian blood cells, can reduce vanadate(V) to vanadyl(IV) and maintain it in the reduced form.

Introduction

Vanadium, a multivalent metal, is generally present in the biosphere in the +5, +4, and +3 oxidation states (Chasteen, 1983; Kustin *et al.*, 1983). Among all organisms examined, ascidians appear to be the only ones that contain high levels of vanadium. High levels of specifically accumulated vanadium in the blood cells of ascidians are reduced predominantly to the +3 oxidation state, with a small amount of vanadium also present in the +4 oxidation state (Tullius *et al.*, 1980; Dingley *et al.*, 1981; Frank *et al.*, 1986; Lee *et al.*, 1988; Hirata and Michibata, 1990), even though the vanadium dissolved in seawater seems to be present as vanadate(V) anions in the +5 oxidation state (McLeod *et al.*, 1975). Therefore, it has been assumed that some agent that causes the reduction of vanadate(V) to vanadyl(IV) must be present in ascidian blood cells because they accumulate the metal from seawater.

We have already reported the extraction of a vanadium-binding substance, which we have called vanadobin, from the vanadocytes of ascidians (Michibata *et al.*, 1986; Michibata and Uyama, 1990; Michibata *et al.*, 1990a). Vanadium incorporated into vanadobin is maintained in the reduced form and, therefore, vanadobin seems to be able to reduce vanadate(V) to vanadyl(IV). In the present experiments, the metal-reducing ability of vanadobin was examined by spectrophotometry and ESR (electron spin resonance) spectrometry, after we demonstrated that the supernatant of a homogenate of the blood cells could reduce the vanadate(V).

Materials and Methods

Homogenates of blood cells

Specimens of *Ascidia gemmata* were collected at the Asamushi Marine Biological Station of Tohoku University in Asamushi, Aomori, Japan. The animals were transported to our laboratory and maintained in an aerated aquarium at 12°C until use. Blood was collected by cardiac puncture under an anaerobic atmosphere of nitrogen gas to preclude oxidation by air; subsequent manipulations were also carried out under the same conditions. The blood cells were separated from the blood plasma by centrifugation at 3000 × *g* for 10 min at 4°C. About

Received 16 January 1991; accepted 18 March 1991.

* Present address: Mukaishima Marine Biological Laboratory, Hiroshima University, Mukaishima-cho, Hiroshima 722, Japan.

10 g wet weight of the pellet of blood cells were resuspended in 3 ml of acidified, deionized, and distilled water (acidic DDW) that had been degassed, bubbled with nitrogen gas, and adjusted to pH 2.3 with 2 *M* HCl. We feared that the buffer solution, such as HCl-glycine buffer, might interfere with ESR spectrometry; therefore, no buffer solution was used in these experiments. The suspension was then ground in a glass-Teflon homogenizer at 4°C. After adding 12 ml of acidic DDW, the homogenate obtained was centrifuged at 11,500 × *g* for 10 min, to remove the cell debris. An aliquot of the supernatant of the homogenate was examined for its ability to reduce vanadate(V) to vanadyl(IV).

Extraction of vanadobin (vanadium-binding substance)

The extraction of vanadobin was carried out as described previously using 7 ml of supernatant obtained as described above (Michibata *et al.*, 1990a). The supernatant was loaded onto a column (3.6 cm ϕ × 56 cm long) of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with acidic DDW in 5 ml.

The peak fractions, monitored at 254 nm, were separately pooled and lyophilized, and then were redissolved in 10 ml of acidic DDW and kept in anaerobic atmosphere before use.

Spectrophotometric measurements of the reduction of vanadate(V)

Aqueous solutions of vanadium(III) sulfate [$V_2(SO_4)_3$] and vanadium(IV) oxide sulfate ($VOSO_4$) at low pH exhibit absorption maxima at 420 nm and 620 nm, and at 760 nm with a shoulder around 625 nm, respectively, whereas an aqueous solution of sodium vanadium(V) (Na_3VO_4) exhibits no absorption maximum in the visible range. We have already demonstrated that the ratio of vanadium ions in the +3 state to those in the +4 oxidation state, which are associated with vanadobin, can be calculated from the respective molar absorption coefficients (ϵ) (Michibata *et al.*, 1990a), using the following formulae:

$$D_{620} = A[M] \times \epsilon^{III}{}_{620} + B[M] \times \epsilon^{IV}{}_{620}$$

$$D_{760} = A[M] \times \epsilon^{III}{}_{760} + B[M] \times \epsilon^{IV}{}_{760}$$

Here, D_{620} and D_{760} are the observed absorbance of vanadobin at 620 nm and 760 nm, respectively and $\epsilon^{III}{}_{620}$, $\epsilon^{III}{}_{760}$, $\epsilon^{IV}{}_{620}$, and $\epsilon^{IV}{}_{760}$ are the molar absorption coefficients of inorganic vanadium(III) and vanadium(IV) in water at each wavelength. Molar concentrations of vanadium(III) and vanadium(IV) associated with vanadobin, A[M] and B[M], can be calculated from the observed absorbance at 620 nm and 760 nm.

Electron spin resonance (ESR) measurements of the reduction of vanadate(V)

ESR spectrometry was carried out as described previously (Hirata and Michibata, 1990). Briefly, 100 μl of a mixture of two volumes of sample and one volume of 4 *M* H_2SO_4 were put into a quartz tube. We used a JES-RE1X ESR spectrometer (JEOL Ltd., Tokyo) for ESR spectrometry. The instrument conditions were adjusted as follows: microwave frequency, 9.2 GHz; magnetic field, 330 ± 100 mT; microwave power, 5 mW; field modulation frequency, 100 kHz; field modulation width, 0.63 mT; and sweep time for recording, 4 min.

Chemicals

All chemicals used were obtained from commercial sources and were of special grade, except for $V_2(SO_4)_3$, which was prepared according to the literature (Claunch and Jones, 1963).

Results

Reduction of vanadate(V) by a homogenate of blood cells

As shown in Figure 1, the supernatant of the homogenate of blood cells exhibits two absorption maxima at

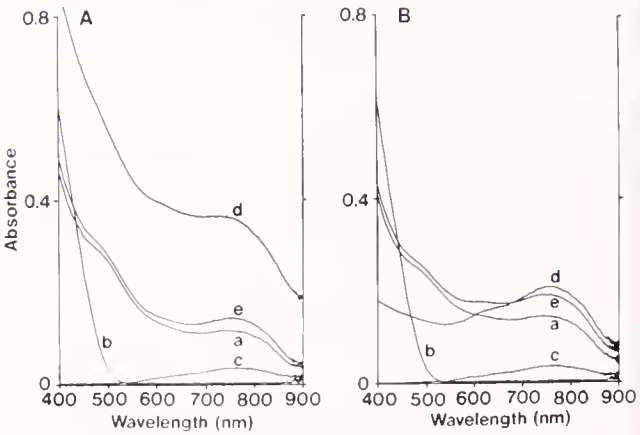

Figure 1. Reduction of vanadate(V) by the supernatant of a homogenate of blood cells from *Ascidia gemmata*, as monitored by spectrophotometry. A. Immediately after the start of the reaction. B. Thirty-one hours after the start of the reaction. Absorption spectrum of the supernatant of the homogenate of blood cells (a), of 8 m*M* vanadate(V) (b), and of 8 m*M* vanadyl(IV) (c). When an 8 m*M* solution of vanadate(V) was added to the supernatant, absorbance in the vicinity of 760 nm due to vanadyl(IV) increased (d). Thirty-one hours after the reaction, absorbance at the shorter wavelength than 530 nm due to vanadate(V) decreased and that at 760 nm became clear (d), suggesting that the added vanadate(V) was reduced to vanadyl(IV) by the supernatant. By contrast, addition of an 8 m*M* solution of vanadyl(IV) to the supernatant resulted in little change in the absorption spectrum (e) both immediately after and 31 hours after the start of the reaction. The higher base line in the absorption spectrum of (d) than the others caused turbidity appeared when vanadate(V) solution was added to the sample.

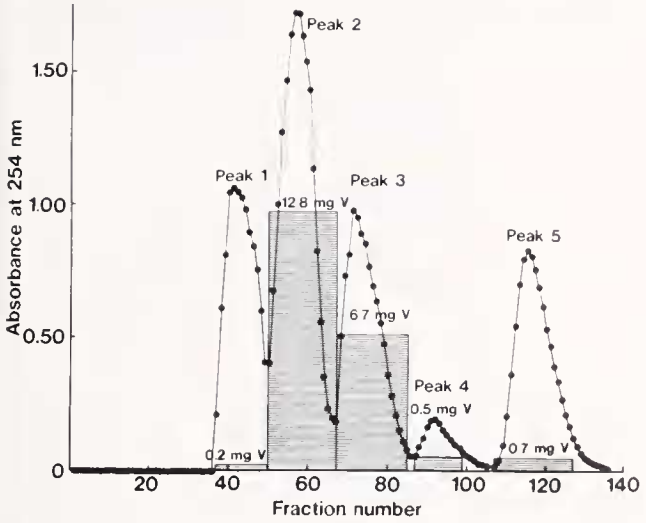

Figure 2. Elution profile of the supernatant of a homogenate of blood cells from *Ascidia gemmata* from a column of Sephadex G-15. The supernatant (7 ml) was loaded onto a column (3.6 cm $\phi \times 56$ cm long) of Sephadex G-15 (Pharmacia Fine Chemicals) and eluted with DDW (see text) at pH 2.3 in 5 ml. Fractions composing each peak were separately pooled, and amounts of vanadium in individual aliquots were measured by ESR spectrometry. Then, the material under each peak was lyophilized and kept under anaerobic conditions. The material in the second peak contained the highest amount of vanadium. V_t: 570 ml, V_o: 226 ml, and V_e: 280 ml.

620 nm and 760 nm, which are assignable to vanadium(III) and vanadyl(IV), respectively. Comparing the corresponding spectra observed in inorganic vanadium complexes, as demonstrated previously (Michibata *et al.*, 1990a), we calculated that the supernatant of the homogenate of the blood cells intrinsically contained 25 m*M* vanadium in the +3 and +4 oxidation states at a ratio of 30:70.

After adding 1.2 ml of an 8 m*M* solution of vanadate(V) to an equal volume of the supernatant, spectral changes were recorded. Figures 1A and 1B show the spectra immediately after mixing and after 31 h. The absorbance at 760 nm due to vanadyl(IV) became conspicuous, accompanying the decrease of the absorbance at the wavelength shorter than 530 nm due to vanadate(V) with time, suggesting that the added vanadate(V) was reduced to vanadyl(IV) by the supernatant. By contrast, the addition of the same amount of an 8 m*M* solution of vanadyl(IV) resulted in little change. These observations indicate clearly that some reducing agent is present in the supernatant of homogenate of blood cells from *Ascidia gemmata*, which can reduce vanadate(V) to vanadyl(IV). As shown in Figure 1B, the spectral change with time reveals that reduction of vanadate(V) to vanadyl(IV) occurred very slowly over the course of 31 h after the onset of the reaction.

Reduction of vanadate(V) by vanadobin: observations by spectrophotometry

When the supernatant of the homogenate of blood cells was eluted from Sephadex G-15, five peaks were obtained (Fig. 2). Amounts of vanadium contained in each peak of material are illustrated as shaded squares in Figure 2.

Reduction experiments were performed with samples of elutant that had been lyophilized and redissolved in 10 ml of acidic DDW. From the ratio of absorbance at 620 nm to that at 760 nm, it was calculated that the material in peak 2 contained vanadium in the +3 and +4 oxidation states at a ratio of 1:25. When 0.8 ml of a solution of vanadyl(IV) or vanadate(V) at concentrations from 4 m*M* to 16 m*M* was added to each peak fraction, reduction of vanadate(V) to vanadyl(IV) was significant in the case of the material in peak 2, when monitored by spectropho-

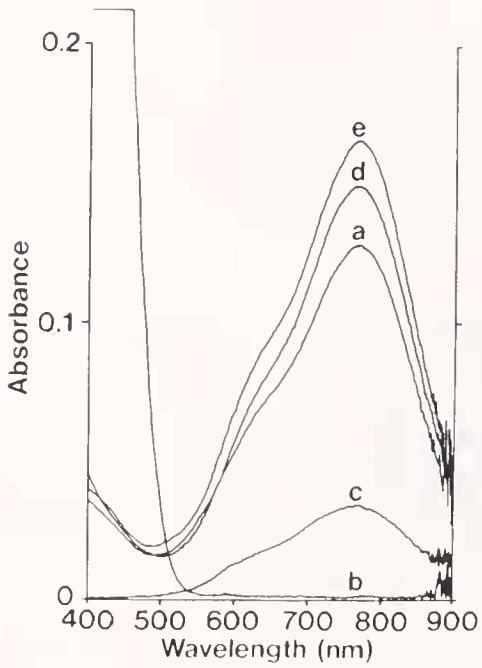

Figure 3. Reduction of vanadate(V) by vanadobin from *Ascidia gemmata* as monitored by spectrophotometry. The samples of lyophilized material (described in the legend to Fig. 2) were redissolved in 10 ml of acidic DDW and reacted with an equal volume of either vanadate(IV) or vanadyl(V), both solutions were at 8 m*M* to examine their ability to reduce vanadium. Absorption spectra of: (a) vanadobin; (b) an 8 m*M* solution of vanadate(V); and (c) an 8 m*M* solution of vanadyl(IV). (d) When vanadate(V) (8 m*M*) was reacted with vanadobin, a marked increased in absorbance at about 760 nm due to vanadyl(IV) was observed, indicating that vanadate(V) was reduced to vanadyl(IV) by vanadobin. (e) By contrast, addition of vanadyl(IV) (8 m*M*) to vanadobin resulted in little change in the absorption spectrum, indicating that no further reduction of vanadyl(IV) to vanadium(III) had occurred. Unlike the results obtained with the supernatant of the homogenate of blood cells depicted in Figure 1, the above changes in absorbance were observed immediately after the start of the reaction, and the reduction was maintained for at least 24 h.

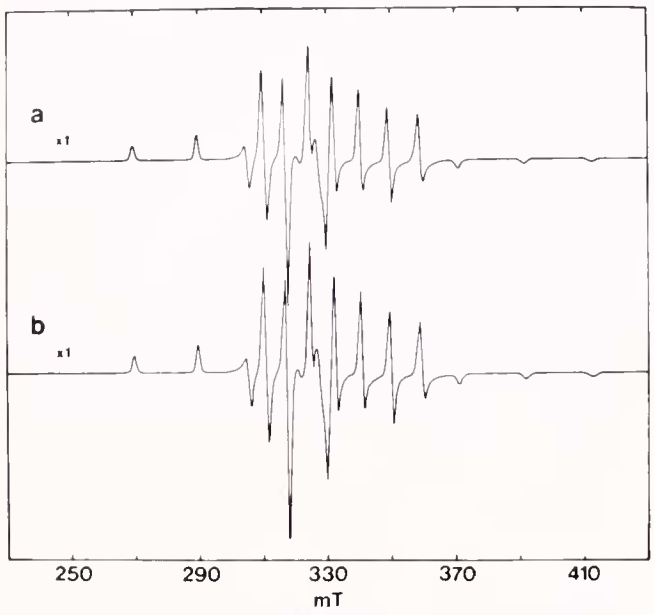

Figure 4. ESR spectra of vanadobin and of a mixture of vanadobin with an 8 m*M* solution of vanadate(V) at 77 K under anaerobic atmosphere of nitrogen gas. (a) Vanadobin, (b) a mixture of vanadobin and 8 m*M* vanadate(V). Oxovanadium [VO^{2+}(IV)] that was intrinsically present in the vanadobin gave typical ESR signals (a). The addition of an 8 m*M* solution of vanadate(V), which alone gave no ESR signal, to an equal volume of vanadobin increased the signal intensity to about 1.2 times that generated by vanadobin alone.

tometry. Figure 3 shows the rapid reduction of vanadate(V) to vanadyl(IV) that followed the addition of the material in peak 2 (vanadobin). Although vanadate(V) in solution exhibits no absorption in the vicinity of 760 nm, the addition of vanadobin induced a drastic increase in absorbance at 760 nm, which indicates the reduction of vanadate(V) to vanadyl(IV). This spectral change was observed immediately after the start of the reaction, and the reduction was maintained for at least 24 h.

Reduction of vanadate(V) by vanadobin: observation by ESR spectrometry

ESR spectrometry was used to confirm the above results. The oxovanadyl chemical species [VO^{2+}(IV)] is the only species of vanadium that is detectable with an ESR spectrometer. Because the intensity of the ESR signal due to VO^{2+}(IV) depends on the pH, the pH of samples was adjusted to 0.21 (the pH at which the highest intensity of signals was obtained) by the adding one half volume of 4 *M* H_2SO_4. Figure 4 shows the ESR spectra derived from (a) vanadobin and (b) from a mixture of vanadobin and 8 m*M* vanadate(V). The signal intensity of the latter was about 1.2 times as strong as that of the former solution, suggesting that a portion of the added vanadate(V) was reduced to the vanadyl(IV) species by vanadobin. The

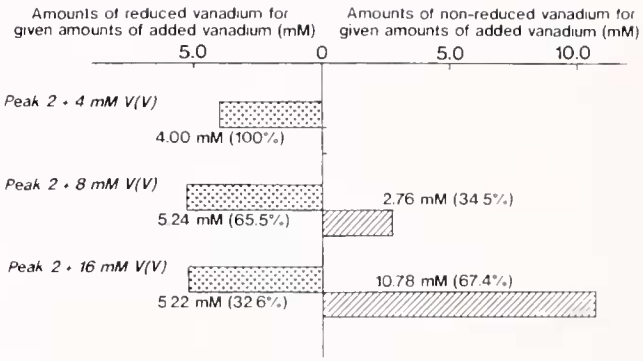

Figure 5. Reduction of vanadate(V) at different concentrations by material from peak 2 contained vanadobin, as measured by ESR spectrometry. All vanadate(V) was reduced to vanadyl(IV) at a concentration of 4 m*M* vanadate(V). However, at 8 m*M* and 16 m*M*, a part of the vanadate(V) added was reduced by vanadobin. Thus, 0.8 ml of solution of vanadobin could completely reduce about 5.2 m*M* of vanadate. In 0.8 ml of the solution of vanadobin, the concentration of vanadium was intrinsically 25 m*M*. If 1 mole of vanadobin contains 1 mole of vanadium ions, then 5 moles of vanadobin readily reduce 1 mole of vanadate(V) to vanadyl(IV).

results obtained are summarized in Figure 5; all of the vanadium in 4 m*M* vanadate(V), 65.5% of that in 8 m*M* vanadate(V) and 32.6% of that in 16 m*M* vanadate(V) was reduced, respectively, after adding vanadobin. It is clear that 0.8 ml of vanadobin prepared by us can reduce 5.2 m*M* vanadate(V) to vanadyl(IV). An aliquot of 0.8 ml of vanadobin contained 25 m*M* intrinsic vanadium. If 1 mole of vanadobin contains 1 mole of vanadium, 5 moles of vanadobin readily reduce 1 mole of vanadate(V) to vanadyl(IV). This assumption, however, needs further investigation.

When the same experiments were carried out with the material in peak 1, a decrease in reducing ability was detected, as shown in Figure 6. The material in peak 1 re-

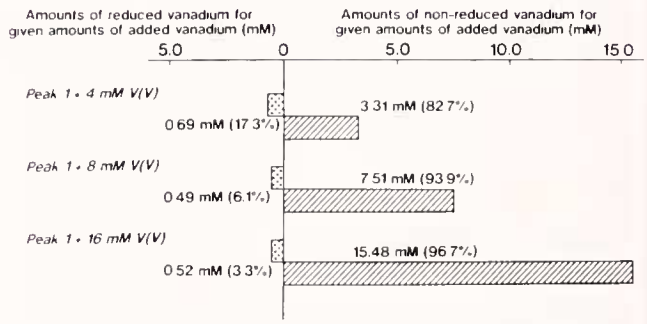

Figure 6. Reduction of vanadate(V) at different concentrations by material in peak 1 which contained no vanadobin, measured by ESR spectrometry as described in the legend to Figure 5. Less reducing ability was observed in this case, unlike that shown in Figure 5. The material in peak 1 reduced a solution of about 0.5 m*M* vanadate(V) which corresponded to between 17.3% and 3.3% of the added metal.

duced about 0.5 m*M* vanadate(V), which corresponded to 17.3% to 3.3% of the added metal. The material in the other peaks eluted from the column showed little reducing ability. Among the rest, the material in peak 3 did not show a reducing ability, although it contained the second highest level of vanadium.

Discussion

Because almost all the vanadium contained in vanadobin is kept in a reduced chemical form (Michibata *et al.*, 1990a), it seems clear that vanadobin cannot only reduce the metal but also maintain in the reduced form. The present results have demonstrated that vanadobin, extracted from the vanadium-rich blood cells of *Ascidia gemmata* by elution from a column of Sephadex G-15, can reduce vanadate(V) to vanadyl(IV), as shown both by spectrophotometry and ESR spectrometry. We have confirmed that no reduction of vanadate(V) occurred when inorganic vanadate(V) and vanadyl(IV) (8 m*M*) were mixed together under the same conditions as those used in the experiments described here (data not shown). It is therefore clear that vanadobin reduces vanadate(V) to vanadyl(IV).

Except for the vanadobin eluted in peak 2 (Fig. 2), the materials in the other peaks showed little ability to reduce the metal. Unexpectedly, the material in peak 3, which contained the second highest amount of vanadium (Fig. 2), did not show a reducing ability. Although its absorption spectrum was different from that of peak 2, which was the typical spectrum of vanadobin, it closely resembled that of the inorganic vanadium complex in the +4 oxidation state, as reported previously (Michibata *et al.*, 1990a). In other words, it may be that peak 3 did not contain vanadobin but inorganic vanadium released from vanadobin; therefore, no reducing ability would be observed.

A crude supernatant, obtained from a homogenate of the blood cells of *A. gemmata,* was also able to reduce vanadate(V). This result is not surprising. In living blood cells, various reducing agents, such as ascorbic acid, glutathione, and cysteine, are generally present. In fact, the supernatant reduced vanadate(V) slowly—for over 31 h after mixing of the supernatant with vanadate(V)—while the extracted vanadobin rapidly reduced the metal after mixing vanadobin with vanadate(V). These phenomena suggest that the crude supernatant contains not only several reducing agents but also several oxidizing agents and, therefore, the reduction of vanadate(V) to vanadyl(IV) may compete against a re-oxidation of vanadium(IV) by some intrinsic oxidizing agents. This may require much more time than that required for purer vanadobin.

Nakanishi's group isolated a tunichrome, composed of three pyrogallol subunits, from ascidian blood cells, and they proposed that it was involved in both the accumulation and the reduction of vanadium in the blood cells (Macara *et al.*, 1979a, b; Bruening *et al.*, 1985). However, in addition to the fact that no fluorescence due to the tunichrome was observed from the vanadocytes (Michibata *et al.*, 1988; 1990b), Bulls *et al.* (1990) pointed out that analogues of the tunichrome were barely able to reduce vanadium(V) to vanadium(IV). Moreover, specific binding of vanadium has not yet been observed within the tunichrome. Therefore, it is noteworthy that the agent that combines with vanadium can reduce vanadate(V), as shown in the present experiments.

We have already demonstrated that the vanadium-containing blood cells—the vanadocytes—are the signet ring cells, and not the morula cells as previously thought (Michibata *et al.*, 1987); we have also shown that vanadobin is contained in the signet ring cells (Michibata and Uyama, 1990) and we have demonstrated that vanadobin may well be a universal complex in ascidians, playing a prominent role in the accumulation of vanadium in blood cells and in the maintenance of its concentration (Michibata *et al.*, 1990a). Therefore, we conclude that vanadate(V) is reduced by vanadobin in the vanadocytes of vanadium-containing ascidians, even though details of the mechanism remain unresolved. The physiological ability, observed uniquely in ascidian blood cells, to reduce and maintain vanadium in the low-oxidation state attracts many investigators in a variety of fields.

Acknowledgments

We would like to express our heartfelt thanks to Dr. T. Numakunai and all other members of the staff of the Asamushi Marine Biological Station of Tohoku University in Asamushi, Aomori, Japan, for supplying the materials and facilitating parts of our work. Thanks are also due to Dr. A. Takeuchi of Toyama University for kindly making his ESR spectrometer available to us. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan (#01480026 and #01304007) and was also supported financially from the Institute for Marine Biotechnology.

Literature Cited

Bruening, R. C., E. M. Oltz, J. Furukawa, K. Nakanishi, and K. Kustin. 1985. Isolation and structure of tunichrome B-1, a reducing blood pigment from the tunicate *Ascidia nigra* L. *J. Am. Chem. Soc.* **107**: 5298–5300.

Bulls, A. R., C. G. Pippin, F. E. Hahn, and K. N. Raymond. 1990. Synthesis and characterization of a series of vanadium-tunichrome B 1 analogues. Crystal structure of a tris(catecholamide) complex of vanadium. *J. Am. Chem. Soc.* **112**: 2627–2632.

Chasteen, N. D. 1983. The biochemistry of vanadium. *Structure and Bonding* **53**: 105–138.

Claunch, R. T., and M. M. Jones. 1963. Vanadium(III) sulfate. *Inorg. Synthesis* **7**: 92–94.

Dingley, A. L., K. Kustin, I. G. Macara, and G. C. McLeod. 1981. Accumulation of vanadium by tunicate blood cells occurs via a specific anion transport system. *Biochim. Biophys. Acta* **649:** 493–502.

Frank, P., R. M. K. Carlson, and K. O. Hodgson. 1986. Vanadyl ion EPR as a noninvasive probe of pH in intact vanadocytes from *Ascidia ceratodes*. *Inorg. Chem.* **25:** 470–478.

Hirata, J., and H. Michibata. 1990. Valency of vanadium in the vanadocytes of *Ascidia gemmata* separated by density-gradient centrifugation. *J. Exp. Zool.* **257:** 160–165.

Kustin, K., G. C. McLeod, T. R. Gilbert, and L. B. R. Briggs. 1983. Vanadium and other metal ions in the physiological ecology of marine organisms. *Structure and Bonding* **53:** 139–160.

Lee, S., K. Kustin, W. E. Robinson, R. B. Frankel, and K. Spartalian. 1988. Magnetic properties of tunicate blood cells. I. *Ascidia nigra*. *J. Inorg. Biochem.* **33:** 183–192.

Macara, I. G., G. C. McLeod, and K. Kustin. 1979a. Isolation, properties and structural studies on a compound from tunicate blood cells that may be involved in vanadium accumulation. *Biochem. J.* **181:** 457–465.

Macara, I. G., G. C. McLeod, and K. Kustin. 1979b. Tunichromes and metal ion accumulation in tunicate blood cells. *Comp. Biochem Physiol.* **63B:** 299–302.

McLeod, G. C., K. V. Ladd, K. Kustin, and D. L. Toppen. 1975. Extraction of vanadium(V) from seawater by tunicates: a revision of concepts. *Limnol. Oceanogr.* **20:** 491–493.

Michibata, H., T. Miyamoto, and H. Sakurai. 1986. Purification of vanadium binding substance from the blood cells of the tunicate, *Ascidia sydneyensis samea*. *Biochem. Biophys. Res. Commun.* **141:** 251–257.

Michibata, H., J. Hirata, M. Uesaka, T. Numakunai, and H. Sakurai. 1987. Separation of vanadocytes: determination and characterization of vanadium ion in the separated blood cells of the ascidian, *Ascidia ahodori*. *J. Exp. Zool.* **244:** 33–38.

Michibata, H., J. Hirata, T. Terada, and H. Sakurai. 1988. Autonomous fluorescence of ascidian blood cells with special reference to identification of vanadocytes. *Experientia* **44:** 906–907.

Michibata, H., H. Hirose, K. Sugiyama, Y. Ookubo, and K. Kanamori. 1990a. Extraction of a vanadium-binding substance (vanadobin) from the blood cells of several ascidian species. *Biol. Bull.* **179:** 140–147.

Michibata, H., and T. Uyama. 1990. Extraction of vanadium-binding substance (vanadobin) from a subpopulation of signet ring cells newly identified as vanadocytes in ascidians. *J. Exp. Zool.* **254:** 132–137.

Michibata, H., T. Uyama, and J. Hirata. 1990b. Vanadium-containing blood cells (vanadocytes) show no fluorescence due to the tunichrome in the ascidian, *Ascidia sydneyensis samea*. *Zool. Sci.* **7:** 55–61.

Tullius, T. D., W. O. Gillum, R. M. K. Carlson, and K. O. Hodgson. 1980. Structural study of the vanadium complex in living ascidian blood cells by X-ray absorption spectrometry. *J. Am. Chem. Soc.* **102:** 5670–5676.